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For oral communications with more than one author, the first author denotes the one who presented the work.

COMMUNICATIONS

Differentiation of compounds which bind to P450 isozymes CYP2C9 and CYP3A4 by physicochemical properties

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The cytochrome P450 isozymes CYP3A4 and CYP2C9 are responsible for many of the xenobiotic oxidations which occur in man. CYP2C9 shows a high degree of structural specificity, with substrates characterised by a region of hydrophilicity and strong hydrogen bond forming potential, e.g. the sulphonylurea of tolbutamide [1]. In contrast, CYP3A4 shows little structural selectivity, being responsible for the oxidation of a diverse series of chemical entities (e.g. steroids, dihydropyridines, macrolides). The common feature for CYP3A4 substrates is that they are all lipophilic [2] and have substituents labile to electron or hydrogen atom abstraction.

Based on lipophilicity measured by octanol/buffer partitioning there is a degree of overlap between the substrates of CYP2C9 and CYP3A4; e.g. the CYP2C9 substrate, tolbutamide with a log $D_{7.4(octanol)}$ value of 0.52 would generally be considered sufficiently lipophilic to be a substrate for CYP3A4. Moreover, tolbutamide has a benzylic methyl function which is favourable for metabolism by CYP3A4 and is the site of metabolism by CYP2C9. We have therefore tried to rationalise the selectivity of the isozymes, in particular the exclusion of CYP2C9 substrates from CYP3A4, by further refinement of physicochemical measurement.

Compounds can partition into the octanol phase of an octanol/buffer mixture associated with their water molecules of hydration. When liphophilicity is determined in cyclohexane (CHX), a solvent that does not support hydrogen bonding, only the drug molecule can partition into the organic phase and not the hydrated molecule. In this system phenytoin appears markedly more hydrophilic than it does in an octanol system with a log $D_{7.4(CHX)}$ value of −2.01. Indeed two other molecules known to bind to CYP2C9, tolbutamide (a substrate) and sulphaphenazole (a specific inhibitor) also have log $D_{7.4(CHX)}$ values below −2.0. In contrast, five substrates of CYP3A4 remain highly lipophilic when measured in such a system, with values ranging from 1.00 to 3.53 (Table 1). Ketoconazole, a CYP3A4 inhibitor, does appear markedly more hydrophilic with a Δ log D (log $D_{7.4(octanol)}$ − log $D_{7.4(CHX)}$) of 4.5, but remains more lipophilic than the CYP2C9 substrates and inhibitors.

These experiments therefore provide a model system for understanding the substrate/inhibitor selectivity of CYP2C9 and CYP3A4. Our present hypothesis is that the lipophilic binding site of CYP3A4 requires molecules to shed their sphere of hydration. In contrast, binding to CYP2C9 is much more tolerant of the degree of hydration.

Table 1 Liphophilicity values of cytochrome P450 substrates and inhibitors determined in octanol and cyclohexane

Compound	*log $D_{7.4(octanol)}$*	*log $D_{7.4(CHX)}$*	*Δ log D*
Phenytoin	2.40	−2.01	4.41
Tolbutamide	0.52	−2.41	2.93
Sulphaphenazole	0.22	−4.12	4.34
Erythromycin	1.95	2.15	−0.20
Felodipine	3.91	2.74	1.17
Ketoconazole	3.86	−0.64	4.50
Terfenadine	5.18	3.53	1.65
Testosterone	3.51	1.00	2.51
Verapamil	3.15	1.79	1.36

1 Jones BC, *et al. Br J Clin Pharmacol* 1993; **36**: 143P.

2 Smith DA, Jones BC. *Biochem Pharmacol* 1992; **44**: 2089.

Catalytic functions of aspartic acid 301 in debrisoquine 4-hydroxylase cytochrome P450 (CYP2D6)

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Cytochrome P450 2D6 (CYP2D6) is responsible for the metabolism of many drugs in clinical use. Although structurally diverse, all CYP2D6 substrates possess a basic nitrogen which is presumed to be protonated. Molecular modelling studies have generated substrate-template models for CYP2D6 [1–3] which predict an interaction between the basic nitrogen atom of the substrate and a negatively charged site in the protein, such as the carboxylate anion of aspartate or glutamate. This interaction is implicated in the mechanism of CYP2D6 catalysis. A candidate amino acid for such an electrostatic interaction is aspartic acid (Asp) 301 [2, 4]. In order to investigate the role

of Asp 301, we substituted this residue in human CYP2D6 with either alanine (Ala), asparagine (Asn) or glutamic acid (Glu)), amino acids which differ in size, charge and polarity. We subsequently assessed the effects of these substitutions on protein expression, stability and catalytic activity using a yeast heterologous expression system [5]. Wildtype (Asp 301) and each substitution resulted in comparable levels of expressed protein as quantified by western immunoblotting. However, the levels of holoprotein (heme + apoprotein), as detected by CO-difference spectroscopy, differed markedly depending on the substituent (Glu: 113%; Asn: 60% and Ala: 10% of wild type) (Table 1). Catalytic activity, as assessed by α-hydroxylation (αOH) and *O*-demethylation (ODM) of metoprolol, was virtually abolished when Asp was replaced by Ala (tiny, uncharged, hydrophobic residue) or Asn (small, uncharged, polar residue), whereas catalytic activity was retained with Glu (negatively charged, polar residue; similar to aspartic acid but extended by a methylene group) (Table 1). However, the regioselective metabolism of metoprolol (ODM:αOH ratio) was altered in the Glu mutant (Table 1). Thus the nature of the amino acid residue at position 301 markedly influences the structure of CYP2D6, a strict requirement for a negatively charged, polar residue being paramount for stability and catalytic activity. The results support the proposition that aspartic acid 301 is involved in catalysis via an electrostatic interaction with the basic nitrogen of CYP2D6 substrates.

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Table 1 Expression and catalytic activity of wild type and mutant forms of CYP2D6

CYP2D6	*P450 expressed as holoprotein**	*Metoprolol metabolism†* αOH	ODM
Wild type	64 ± 3	0.62 ± 0.03	2.31 ± 0.02
301 Glu	72 ± 2	0.27 ± 0.04	2.13 ± 0.27
301 Asn	39 ± 6	0.02 ± 0.01	0.07 ± 0.01
301 Ala	7 ± 2	0.02 ± 0.01	0.04 ± 0.01

Values are means ± s.d., n = 3; *pmol mg^{-1} protein; †pmol min^{-1} $pmol^{-1}$ P450.

1 Wolff T, *et al. Cancer Res* 1985; **45**: 2116.
2 Islam SA, *et al. Carcinogenesis* 1991; **12**: 2211.
3 Koymans LMH, *et al. Chem Res Toxicol* 1992; **5**: 211.
4 Koymans LMH, *et al. J Comput-Aided Mol Design* 1993; **7**: 281.
5 Ellis SW, *et al. Biochem Pharmacol* 1992; **44**: 617.

Production of a human CYP3A antibody from multiple peptide sequences

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CYP3A is the major P450 isozyme present in human liver. This isozyme is also one of the major drug metabolising enzymes and is responsible for the metabolism of a variety of compounds such as erythromycin, nifedipine, terfenidine and cyclosporin A [1]. Due to the importance of this isozyme in xenobiotic metabolism we wished to characterise a human liver bank for both CYP3A protein and activity. The aim of this work was to raise a Western Blotting antibody to this isozyme from a multiple peptide sequence thereby dispensing with the need for purified enzyme as the antigen source.

To this end a series of multiple peptides were prepared (Table 1). The peptide sequences were derived from unique parts of the human CYP3A4 primary sequence and synthesised as 8-branched MAPs peptides. The peptides were mixed together and used to immunise two rabbits from which plasma was collected.

Table 1 Peptide sequences for raising CYP3A antibody

Peptide sequence	*Amino acid residues*
IPDLAMETW	4–12
YGTHSHGLFK	25–34
SYHKGFCMFDMECH	52–65
AITDPDMIKTV	83–93
YSVFTNRRPFGPVGFM	99–114
KEMVPIIAQYGDV	143–155
REAETGKPVTLKD	162–174
VNIDSLNNPQDPFV	191–204
EVLNICVFPRE	234–244
RLEDTQKHRV	260–269
LQLMIDSQNSKE	272–283
AVLPNKAPPTYDTV	337–351
LFPIAMRLERVCKK	366–379
MIPSYALHRD	395–404
SKKNKDNIDPYTYT	420–433
PCKETQIPLKLSLQ	467–484 (–4aas)
LKVESRDGTVSGA	491–503

The high titre sera recognised a single band on Western Blot analysis of human and rat liver microsomes. This band corresponded to the intense signal produced with dexamethasone-induced rat liver microsomes. The antibody was used to quantitate protein in a bank of 12 human livers using Western Blotting techniques. The relative amounts of CYP3A protein were then correlated with the CYP3A activity in each liver using probe assays. Testosterone 6β-hydroxylase activity correlated with CYP3A protein with a correlation coefficient of 0.89. Felodipine oxidase activity was also correlated with a value of 0.79. There was poor correlation ($r < 0.02$) with phenacetin *O*-deethylase (CYP1A2), phenytoin 4-hydroxylase (CYP2C9), bufuralol 1'-hydroxylase (CYP2D6) or chlorzoxazone 6-hydroxylase (CYP2E1) activity.

This is the first example, to our knowledge, of the preparation of an antibody that recognises human CYP3A using multiple peptide sequences chosen from conserved regions of the human CYP3A4 protein. It is suitable for quantitation of the isoenzyme by Western Blotting techniques.

1 Smith DA, Jones BC. *Biochem Pharmacol* 1992; **44**: 2089.

Epoxidation, diol formation and glutathione conjugation in rat hepatocytes and precision-cut liver slices

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We have shown previously that quantitative differences exist between freshly isolated rat hepatocytes and precision-cut liver slices in terms of testosterone and 7-ethoxycoumarin metabolism, possibly due to metabolism being confined to the outer layers of hepatocytes in slices [1]. This work was extended by using 1-chloro-2,4-dinitrobenzene (CDNB) as a non-specific marker for glutathione S-transferases and styrene (STY) and carbamazepine (CBZ) to study linkage of cytochrome P450 with epoxide hydroxylase. Additionally we used 2-(5'-chloro-2'-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone (CPPCQ), a fluorescent histochemical substrate for alkaline phosphatase, to compare the rates of diffusion into slices and hepatocytes.

Precision-cut liver slices were prepared from male Sprague Dawley rats (250–300 g). Duplicate vials containing one slice per mesh were incubated with substrates (CDNB 50 μM, STY 2 mM or CBZ 50 μM) in Krebs-HEPES (5 ml), pH 7.4 at 37° C.

Isolated hepatocytes, initial viability >90%, were incubated with substrates at 10^6 cells ml^{-1} in 95% O_2/5% CO_2. STY and CBZ metabolism was measured by h.p.l.c. analysis of metabolites in the medium. The formation of CDNB glutathione conjugate in the medium was measured by the change in absorbance at 320 nm [2].

CBZ and STY metabolism in precision-cut liver slices and hepatocytes confirms CBZ as a poor substrate for epoxide hydrolase in the rat (Table 1, [3]), whilst STY is totally converted to the diol in the rat as observed previously in microsomes and hepatocytes [4]. Fluorescence histochemistry using CPPCQ demonstrated rapid uptake in isolated hepatocytes whereas only the outer 2–3 layers of hepatocytes in slices showed staining at 1 h, possibly explaining the quantitative differences in metabolism [5].

Table 1 Metabolite formation (pmol min^{-1} mg^{-1} protein ± s.e. mean) CDNB incubated for 3 min, STY for 30 min, CBZ for 20 min, $n = 3$

	CBZ epoxide	*CBZ diol*	*Styrene epoxide*	*Styrene diol*	*GSH-CDNB*	*GSH content (nmol/10^6 cells)*
Slices	0.27 ± 0.1	NQ	NQ	16.6 ± 3.93	542 ± 172	35.5
Hepatocytes	0.65 ± 0.09*	NQ	NQ	165 ± 43.1*	4700 ± 268*	30

NQ = not quantifiable, *$P < 0.05$ when compared by Student's unpaired *t*-test.

1 Ekins S, *et al. Br J Clin Pharmacol* 1994; **38**: 164P.
2 Habig WH, *et al. J Biol Chem* 1974; **249**: 7130.
3 Pachecka J, *et al. Xenobiotica* 1976; **6**: 593.
4 Belvedere G, *et al. Toxicol Lett* 1984; **23**: 157.
5 Dogterom P. *Drug Metab Dispos* 1993; **21**: 699.

Induction of diazepam clearance *in vitro* in rat by dexamethasone: quantitative predictions from microsomal and hepatocyte studies

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Diazepam (DZ) is a known substrate for various cytochrome P450 isoforms (CYP); dexamethasone (DEX) is widely used to induce the CYP3A subfamily. In a previous study we investigated the *in vitro* metabolite kinetics of DZ in microsomes and hepatocytes prepared from untreated (UT) and DEX treated rats [1]. The aim of the work described herewith is to determine the *in vivo* intrinsic clearance (CLint) of DZ in UT and DEX rats and hence to assess the predictive value of our earlier determined *in vitro* data.

DZ (10 mg kg^{-1}) was administered to UT (n = 6) and DEX treated (80 mg kg^{-1} in 2% Tween 80, n = 5) male Sprague-Dawley rats (250 g, standard rat weight (SRW), unanaesthetised, previously cannulated) by the hepatic portal vein, via the lineal vein, over a 5 min period. Blood samples were taken at various time intervals up to 90 min and plasma separated by centrifugation. Plasma samples were analysed by reverse-phase h.p.l.c. with u.v. detection. Data are presented as mean ± s.d.

In DEX rats compared with UT rats, the terminal half-life decreased significantly (20.7 ± 17.0 and 58.2 ± 57.4 min respectively, $P < 0.05$), clearance increased significantly (0.283 ± 0.094 and 0.139 ± 0.057 l min^{-1} kg^{-1} respectively, $P < 0.05$) whilst the volume of distribution remained unaltered (3.0 ± 1.1 and 5.1 ± 1.8 l kg^{-1} respectively, $P > 0.05$). There was no significant difference between vehicle treated rats (2% Tween 80) and UT rats. CLint was calculated from the ratio of CL to the unbound fraction of DZ in plasma (0.22 ± 0.04, concentration independent, by ultracentrifugation) since the blood/plasma ratio was unity. CLint values thus obtained were 158 ± 65.6 and 322 ± 108 ml min^{-1} SRW^{-1} for UT and DEX rats respectively. It can be concluded that DEX causes an increase in CLint *in vivo* as a result of CYP induction.

DZ metabolism proceeds via three parallel pathways including the formation of 3-hydroxydiazepam (3HDZ). The microsomal and hepatocyte CLints for DZ reported previously [1] were scaled to *in vivo* using the microsomal protein recovery and hepatocellularity of UT and DEX rats. Microsomal predictions for UT and DEX rats (54 ± 20 and 247 ± 63 ml min^{-1} SRW^{-1} respectively) represent 34 and 77% of the *in vivo* values respectively, the corresponding hepatocyte predictions (95 ± 5 and 287 ± 89 ml min^{-1} SRW^{-1}) represent 59 and 89% of the *in vivo* values respectively. Hepatocytes are more successful than microsomes in predicting the *in vivo* CLint of DZ in UT rats, however a similar level of prediction is seen with DEX rats. In UT cells and microsomes 3HDZ is a minor pathway accounting for 21 and 24% of total DZ metabolism, respectively. However, in DEX cells and microsomes 3HDZ represents 73 and 82% of DZ metabolism, respectively, thus indicating the importance of CYP3A in this pathway. These results suggest that the success of *in vitro* predictions of CLint may be dependent upon the prominence of individual pathways of metabolism and/or the involvement of particular isoenzymes.

1 Carlile DJ, *et al. Br J Clin Pharmacol* 1994; **38**: 177P.

Enzymology of the bioreduction of the pyrazine mono-*N*-oxide RB90740: roles for cytochrome P450 reductase and cytochrome b_5 release

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RB90740 is the lead compound in a series of fused pyrazine mono-*N*-oxide bioreductive agents. It is 20-fold more toxic to hypoxic than to aerobic V79 cells *in vitro*. Its mechanism of toxicity is considered to be via a 1-electron reduction to give a free radical intermediate which causes DNA strand breakage.

The aim of the current work was to elucidate which enzymes catalyse the bioactivation of RB90740 to its 1-electron free radical intermediate and its subsequent stable 2-electron reduced product, RB92816. Studies were performed using microsomes prepared from the livers of female C3H mice. Microsomes (up to 1.2 mg ml^{-1} final protein concentration) were incubated with the parent drug (200 μM) at 37° C under nitrogen for 45 min. The rate of formation of RB92816 was determined using h.p.l.c.

Both NADH (1 mM) and NADPH (1 mM) supported the formation of RB92816, the former at a rate approximately 80% of the latter (NADPH, 1.06 ± 0.27; NADH, 0.72 ± 0.11 nmol RB92816 formed min^{-1} mg^{-1} protein; mean ± s.d., n = 4) but had no additive effect when co-incubated at equimolar concentrations (NADH + NADPH, 0.99 ± 0.18 nmol

RB92816 formed min^{-1} mg^{-1} protein). This indicates that the two cofactors are acting as alternative electron sources for the same enzyme system.

The P450 inhibitors, carbon monoxide and metyrapone (5 mM) did not inhibit NADPH-dependent formation of RB92816. Similarly, NADPH-dependent reduction of RB90740 was not inhibited by methimazole (1 mM) an inhibitor of the flavin-containing monooxygenases.

Pre-incubation of microsomes with anti-P450 reductase antibodies inhibited RB92816 formation by 50%. However, 2'AMP (50 mM) which inhibits P450 reductase by binding at the NADPH binding site did not inhibit formation of RB92816 yet inhibited the NADH-dependent reduction by 40%.

The role of cytochrome b_5 reductase was investigated using *p*-hydroxymercuribenzoate (*p*HMB) and 5-propylthiouracil (PTU) as inhibitors. Both *p*HMB (0.1 mM) and PTU (50 mM), totally inhibited microsomal cytochrome b_5 reductase activity, measured spectrophotometrically as the NADH-dependent reduction of cytochrome c, but did not inhibit microsomal P450 reductase activity, measured spectrophotometrically as the NADPH-dependent reduction of cytochrome c. Both inhibitors completely inhibited both the NADH- and the NADPH-dependent reduction of RB90740 to RB92816.

Our results indicate that both P450 reductase and cytochrome b_5 reductase are involved in the microsomal reduction of RB90740. The enzymes probably do not work independently but in conjunction since inhibition of cytochrome b_5 reductase by pHMB or PTU completely abolished reduction of RB90740, whereas inhibition of P450 reductase by antibodies only partially inhibited RB92816 formation. The most likely explanation is that electrons are transferred by cytochrome b_5 reductase or P450 reductase from NADH or NADPH, respectively to the substrate, *via* cytochrome b_5.

Bioactivation of clozapine *in vitro* and *in vivo*: implications for drug-induced agranulocytosis

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Clozapine (CLZ) is a dibenzodiazepine neuroleptic whose use in schizophrenia is restricted to patients resistant to conventional drugs because of a 1% incidence of agranulocytosis. Drug-induced agranulocytosis has been linked with the metabolic activation of drugs by neutrophils and bone marrow cells to reactive intermediates [1]. CLZ is oxidized by myeloperoxidase to a free radical, which reacts with glutathione (GSH) to form the C6 and C9 GS-CLZ thioether adducts [2]. In this study, we have investigated CLZ bioactivation *in vitro* and *in vivo*.

Human bone marrow cells and peripheral blood neutrophils were each isolated from a healthy donor by density gradient centrifugation. Microsomes were prepared from the liver of an adult female subject [3]. [11-^{14}C]CLZ (2 μM) was incubated with microsomes (2 mg protein ml^{-1}; 1 mM NADPH) or cells (2×10^6 cells ml^{-1}) in the presence or absence of GSH (1 mM) for 1 h at 37° C. The myeloid cells and neutrophils were activated with phorbol myristate acetate (PMA; 10 ng ml^{-1}). Anaesthetized adult male CD1 mice and Wistar rats were given [11-^{14}C]CLZ i.v. (1 mg kg^{-1} and 5 mg kg^{-1}, respectively). Bile was collected via a bile duct cannula. The supernatants of the *in vitro* incubations and the bile were analysed by radiometric h.p.l.c. and h.p.l.c.-mass spectrometry (Quattro II electrospray interface).

Human bone marrow, neutrophils and liver microsomes metabolised CLZ in the presence of GSH to C6 GS-CLZ, the turnover being 11.6 ± 0.9%, 4.8 ± 0.2%, and 13.7 ± 0.3% (mean ± s.d., n = 3), respectively. The adduct was identified by LCMS (at 2 and 200 μM) and comparison with a synthetic standard. Cellular bioactivation was PMA-dependent. CLZ was also metabolised to desmethyl CLZ and CLZ *N*-oxide by the microsomes.

Biliary excretion accounted for 51.7 ± 15.3% (5 h) and 34.0 ± 8.5% (3 h) of administered radioactivity in rats (n = 6) and mice (n = 4), respectively. All the major biliary metabolites were GSH adducts. Mice excreted four isomeric GS-CLZ adducts—including the C6 and C9 adducts—and GS-8-deschloroCLZ. Rats additionally excreted three putative GSH adducts of a phase I metabolite of CLZ.

In conclusion, we have shown that bioactivation is a major pathway in the metabolism of CLZ *in vitro* and *in vivo*. The chemically reactive metabolites might be involved in the agranulocytosis observed with CLZ.

1 Uetrecht JP. *Metab Rev* 1992; **24**: 299.
2 Fischer V, *et al. Mol Pharmacol* 1991; **40**: 846.
3 Pirmohamed M, *et al. J Pharmacol Exp Ther* 1995; (in press).

POSTER-COMMUNICATIONS

Phenytoin metabolism to 5-(4-hydroxyphenyl)-5-phenylhydantoin in the cat is not inhibited by sulphaphenazole

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Phenytoin (PHT) is a well-established drug used in the treatment of partial and generalised tonic-clonic seizures and *status epilepticus*. Metabolism of PHT to 5-(4-hydroxyphenyl)-5-phenylhydantoin (HPPH) is mostly due to cytochrome P4502C9 [1, 2] and is the major elimination pathway in man [3]. However, orthologues of this enzyme in other species have not been well investigated. In particular, the cat is used as a model in epilepsy and in phenytoin-induced gingival overgrowth, but the metabolism of PHT in this mammal has not been well examined. The present study investigated the inhibitory effects of proadifen HCl (SKF525A, a non-specific inhibitor of cytochrome P450 enzymes), sulphaphenazole (a specific inhibitor of P4502C9) and tolbutamide (a specific substrate for P4502C9) on PHT metabolism to HPPH in cat liver microsomes *in vitro*. The results were compared with those obtained from incubations containing rat and human liver microsomes.

Microsomes were prepared from three liver samples of each species according to an established method. Incubations were performed for 30 min at 37° C in a total volume of 600 μl, which contained 0.1 M phosphate buffer pH 7.4, microsomes (4 mg protein ml^{-1}), phenytoin sodium (500 μM) and NADPH (6 mM). The inhibitory effects, relative to control activity, of SKF525A (300 μM), sulphaphenazole (25 μM) and tolbutamide (1.11 mM) were determined.

Control activities were 345.7 ± 62.6, 0.64 ± 0.17 and 32.8 ± 20.0 pmol HPPH formed min^{-1} mg^{-1} protein in rat, cat and human liver microsomes respectively. The activity present in the presence of the test compounds, expressed as a percentage of control activity, is shown in Table 1.

The different inhibitory activity of the compounds in the three microsomal systems *in vitro* suggests that the P450 enzymes responsible for the metabolism of PHT to HPPH in cat liver may be different from that predominating in man. HPPH is a racemic compound and it has been postulated that its two enantiomers are produced by different isoenzymes [4, 5]. In man HPPH is found to be almost exclusively of the S-(−)-configuration [6]. It is possible that the isoenzyme responsible for the production of the R-(+)-HPPH is more active in the cat liver and is less affected by the inhibitors used in this study.

Table 1 Effect of SKF525A, sulphaphenazole and tolbutamide on the rate of HPPH formation in the three hepatic microsomal systems *in vitro*, expressed as a percentage of control (100%) activity ± s.d.

	% of control activity		
	SKF525A	*Sulphaphenazole*	*Tolbutamide*
Rat	1.1 ± 0.7	n.d.	n.d.
Cat	128.1 ± 21.2	98.2 ± 1.3	74.3 ± 5.6
Man	79.3 ± 19.7	16.6 ± 6.4	36.0 ± 26.5

n.d. = not determined.

1 Doecke CJ, *et al. Br J Clin Pharmacol* 1991; **31**: 125.
2 Veronese M, *et al. Biochem J* 1993; **289**: 533.
3 Glazko AJ, *et al. Clin Pharmacol Ther* 1969; **10**: 498.
4 Butler TC, *et al. J Pharmacol Exp Ther* 1976; **199**: 82.
5 Fritz S, *et al. J Pharmacol Exp Ther* 1987; **241**: 615.
6 Dudley KH. In *Epilepsy and the oral manifestations of phenytoin therapy*, ed Hassell TM. Basel: Karger, 1981; 60–69.

The pharmacokinetics and pharmacodynamics of bisphosphonates in rats with retinoid-induced bone resorption

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The pharmacokinetics (PK) of the geminal bisphosphonates (BP) have been well studied [1]. However, the relationship between the PK and the pharmacodynamics (PD) has not been defined. We are attempting to elucidate this relationship for simple bisphosphonic acids using a model of retinoid-induced bone resorption [2] in thyroparathyroidectomised rats. The PD response employed in these experiments was change in serum Ca relative to control animals. The PK data were derived by use of radiolabelled pamidronate (APD) and based on total radioactivity since the compound is not metabolised.

The bone resorption model protocol [2] was modified to prolong the period of hypercalcaemia.

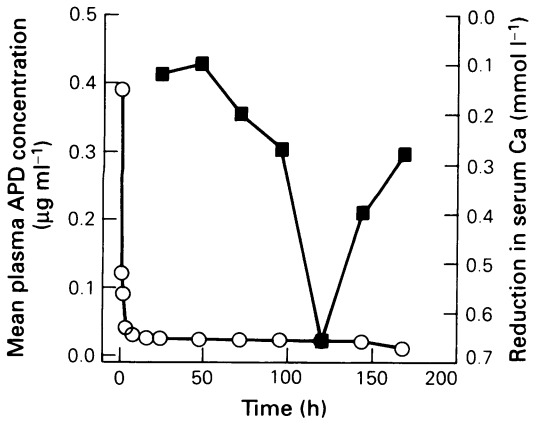

Figure 1 Mean (n = 3) plasma APD concentration (○) and mean effect (■, n = 3) plotted against time following treatment with 0.3 mg kg^{-1} APD.

Dose-response studies defined the optimum single i.v. dose required to inhibit bone resorption by ≈50% to be 0.3 mg kg^{-1} APD. There was an indirect relationship between APD plasma concentration and serum Ca changes (Figure 1). In attempting to describe these data several PK/PD models have been investigated. This indirect relationship could be described by a sigmoid E_{max} PD model linked to an 'effect compartment' PK model. The parameter estimates were: k_{e0} = 0.095 h^{-1}, EC_{50} = 0.042 μg ml^{-1}, E_{max} = 2.2 mM and γ = 51.1. However, it is unlikely that the delayed effect of the drug arises from its distribution dynamics but rather to physiological events related to cell kinetics and calcium redistribution. Therefore, more physiologically based models are being explored.

1 Pongchaidecha M, Daley-Yates PT. *Drug Metab Dispos* 1993; **21**: 100.

2 Trechsel U, *et al. J Clin Invest* 1987; **80**: 1676.

Hepatic expression of mouse PPAR RNAs

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Cytochromes P4504A are induced in rat and mouse liver by peroxisome proliferators, but not in guinea pig [1]. This is accompanied by a wide ranging induction of genes of peroxisomal β-oxidation, and proliferation of endoplasmic reticulum and peroxisomes in the liver. The induction of these genes may be mediated by a novel family of steroid hormone receptors, known as the Peroxisome Proliferator Activated Receptors (PPAR) [2].

The PPAR proteins are not known to bind a specific ligand, nor is it yet clear what their function is. These proteins are 'activated' by peroxisome proliferators, and can induce transcription in model systems, but have an absolute requirement for heterodimer partners (such as Retinoid X Receptor) in order to be functional. The physiological roles, and relevance to peroxisome proliferation, or lipid metabolism, of the three PPARs (α, β and γ) is unclear.

A specific RNAse protection assay was developed to discriminate between the three PPAR RNA transcripts (not shown). This assay was used to investigate the levels of expression of PPAR RNAs in a variety of tissues, including liver. The amount of protected RNA species was determined relative to known amounts of probe, and the number of transcripts per liver cell calculated (Figure 1).

The data indicate that PPARα is the predominant PPAR in liver, with 20-fold less PPARβ RNA present. PPARγ is present at approximately 0.1 molecules per cell, and is unlikely to contribute to hepatic peroxisome proliferation. Similar results were obtained in eight independent experiments.

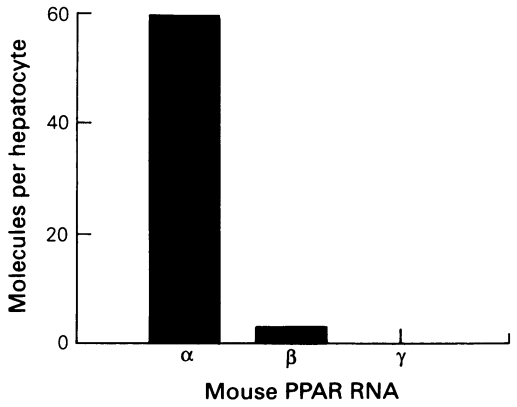

Figure 1

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1 Bell DR, *et al. Biochem J* 1993; **294**: 173.

2 Chen F, *et al. Biochem Biophys Res Commun* 1993; **196**: 671.

The effect of guanamines (cyclised biguanides) on glycogen phosphorylase a activity in hepatocytes from normal rats in different glucose concentrations

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Glucose is a physiological regulator of hepatic glycogen metabolism. It is known that glucose can inactivate glycogen phosphorylase in the liver [1]. Guanamines (cyclised biguanides) have been shown to inhibit glycogen phosphorylase a activity in normal rat hepatocytes suspended in medium containing normal physiological levels of glucose [2]. The purpose of the present work was to investigate the effect of guanamines in the presence of 10 mM (normal) and 30 mM (high) glucose on glycogen phosphorylase a activity in hepatocytes isolated from normal rats. Hepatocytes were isolated from adult, male Wistar rats by the method of Seglen [3] and glycogen phosphorylase was measured according to the method of Stalmans & Hers [4].

The results (Table 1) indicated that guanamines act like insulin (and, at higher concentration, phenformin) in the inactivation of glycogen phosphorylase in 10 mM glucose but increase the activity of the enzyme (as insulin and phenformin do) when the glucose concentration is increased to 30 mM. The most active compound in this series is benzylguanamine in 10 mM and phenylguanamine in 30 mM glucose. From the above findings it can be concluded that the guanamines always show the same effect as insulin (and phenformin) and, thus, can be classed as insulin-mimetics.

Table 1 The effect of insulin, phenformin and guanamines on glycogen phosphorylase a activity in normal hepatocytes incubated with 10 mM and 30 mM glucose

	10 mM			*30 mM*		
Concentration (M)	10^{-9}	10^{-7}	10^{-5}	10^{-9}	10^{-7}	10^{-5}
Insulin	28 ± 2	25 ± 3	24 ± 2	256 ± 19	175 ± 22	252 ± 16
Phenylguanamine	76 ± 5	67 ± 4	43 ± 4	233 ± 41	252 ± 19	269 ± 12
Benzylguanamine	57 ± 3	51 ± 5	49 ± 5	154 ± 18	203 ± 21	294 ± 19
Phenethylguanamine	84 ± 4	80 ± 2	74 ± 4	165 ± 21	214 ± 20	220 ± 17
Phenformin	84 ± 5†	92 ± 2†	85 ± 5*	157 ± 6	156 ± 20	182 ± 11

Values are mean ± s.d. (n = 6), analysed by ANOVA followed by Dunnett's test, as a % of basal phosphorylase activity. Basal phosphorylase activity for 10 mM glucose = 51 ± 3 nmol mg^{-1} protein min^{-1} and for 30 mM = 16 ± 2 nmol mg^{-1} protein min^{-1}.
All values $P < 0.01$, except $^*P < 0.05$, † = not significant.

1 Fleet GWJ, *et al. Biochemistry* 1994; **33**: 5745.
2 Al-Shibani N, Skett P. *Br J Pharmacol* 1994; **113**: 96P.
3 Seglen P. *Meth Cell Biol* 1976; **13**: 29.
4 Stalmans W, Hers HG. *Eur J Biochem* 1975; **54**: 341.

A change in the effect of guanamines on glycogen phosphorylase a activity in hepatocytes from streptozotocin diabetic rats in different glucose concentrations

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Glucose-induced inactivation of glycogen phosphorylase is seen in diabetic rat hepatocytes resulting from the direct interaction of glucose with the enzyme, rendering it a better substrate for phosphorylase phosphatase [1]. Guanamines (cyclised biguanides) have been shown to increase glycogen phosphorylase a activity in hepatocytes from diabetic animals kept in normal glucose concentration (10 mM) as does insulin [2]. The present study examines the effects of guanamines in the presence of 10 mM (normal) and 30 mM glucose (high) on glycogen phosphorylase a activity in hepatocytes isolated from diabetic rats. Hepatocytes were isolated from adult, male Wistar rats made diabetic with streptozotocin by the method of Seglen [3]. Glycogen phosphorylase was measured according to the method of Stalmans & Hers [4].

The results (Table 1) show that insulin, phenformin and guanamines significantly decrease the activity of glycogen phosphorylase a in 30 mM glucose, a result opposite to that found in 10 mM glucose. The most potent compounds in this series were phenethylguanamine in 10 mM and benzylguanamine in 30 mM glucose. The effect is opposite to that seen in hepatocytes from normal rats [5]. This result suggests that guanamines act as insulin mimetics in potentiating the effect of high glucose concentrations on the glycogen phosphorylase in diabetic cells. The reasons for this are unclear.

Table 1 The effect of insulin, phenformin and guanamines on glycogen phosphorylase a activity in diabetic hepatocytes incubated with 10 mM and 30 mM glucose

	10 mM			*30 mM*		
Concentration (M)	10^{-9}	10^{-7}	10^{-5}	10^{-9}	10^{-7}	10^{-5}
Insulin	186 ± 6	203 ± 13	266 ± 6	42 ± 6	39 ± 1	37 ± 5
Phenylguanamine	121 ± 6	110 ± 2	121 ± 4	88 ± 4*	65 ± 10	49 ± 6
Benzylguanamine	69 ± 2	132 ± 3	147 ± 3	56 ± 8	56 ± 5	46 ± 4
Phenethylguanamine	127 ± 8	135 ± 8	136 ± 5	69 ± 6	61 ± 9	55 ± 3
Phenformin	110 ± 6†	121 ± 7	127 ± 4	70 ± 7	67 ± 6	66 ± 5

Values are mean ± s.d. (n = 6), analysed by ANOVA followed by Dunnett's test, as a % of basal phosphorylase activity. Basal phosphorylase activity in 10 mM glucose = 23 ± 4 nmol mg^{-1} protein min^{-1} and 30 mM = 14 ± 2 nmol mg^{-1} protein min^{-1}.
All values $P < 0.01$, except $^*P < 0.05$, † = not significant.

1 Ciudad CJ, *et al. Arch Biochem Biophys* 1988; **267**: 437.
2 Al-Shibani N, Skett P. *Br J Pharmacol* 1994; **113**: 97P.
3 Seglen PP. *Meth Cell Biol* 1976; **13**: 29.
4 Stalmans W, Hers HG. *Eur J Biochem* 1975; **54**: 341.
5 Al-Shibani N, Skett P. *Br J Clin Pharmacol* 1995; **40**: 185P.

The effect of DMSO on steroid metabolism in cultured rat hepatocytes in the presence of hydrocortisone and insulin

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Suspensions of hepatocytes are being used in an increasing number of investigations, including studies on cytochrome P450 content and activity. However, a major concern is the loss in differentiated function, and various attempts to circumvent this problem have been made [1]. This study describes work on the effect of hydrocortisone and insulin in Williams' E medium supplemented with DMSO (2% v/v) on the maintenance of specific cytochrome P450 isoforms in rat hepatocytes. Hepatocytes were isolated from male, Wistar rats using the method of Seglen [2]. Metabolic activity was measured using [^{14}C]-androstenedione as a substrate. The cells were cultured for 24 and 48 h.

After 24 h in culture in the presence of 1 μM hydrocortisone and insulin the activity of 17-OHSD and 5 α-reductase fell significantly compared with control, that of 6β- and 16α-hydroxylase was unchanged and 7α-hydroxylase increased. However after 48 h in culture, the enzyme activity increased significantly as compared with control with the exception of 6β-hydroxylase. DMSO is more effective at maintaining the activity of these enzymes over longer periods in culture.

Table 1 The effect of insulin (In) and hydrocortisone (H.C.) on the androstenedione metabolism in cultured rat hepatocytes

Treatment	*Time (h)*	*7α*	*6β*	*16α*	*17-OHSD*	*5α*
Control	24	30.2 ± 6.1	59.5 ± 8.7	59.7 ± 10.9	87.5 ± 8.2	141.4 ± 15.3
H.C./In 1 μM	24	43.1 ± 5.5*	48.1 ± 2.9	54.2 ± 8.9	42.8 ± 3.6*	47.8 ± 10.3*
Control	48	22.6 ± 2.3	30.3 ± 6.2	32.3 ± 3.2	19.8 ± 2.7	19.6 ± 3.0
H.C./In 1 μM	48	29.3 ± 2.5*	30.5 ± 4.2	39.7 ± 3.2*	32.4 ± 4.8*	35.8 ± 6.7*

Values are expressed as % of zero time control ± s.d. (n = 5); $^*P < 0.01$ compared with respective control (Student's t-test).
The enzymic activities for 7α, 6β, 16α, 17-OHSD and 5α-reductase at zero time were: #10.8 ± 1.8, 18.6 ± 1.7, 23.6 ± 2.6, 62.0 ± 2.7, 211.2 ± 7.1 pmol min^{-1} 10^6 cells.

1 Skett P. *Toxic in vitro* 1994; **8**: 491.
2 Seglen PO. *Meth Cell Biol* 1976; **13**: 29.

Toxicity of allylamine and acrolein towards human cultured endothelial cells: involvement of semicarbazide-sensitive amine oxidase

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The administration of the industrial aliphatic amine allylamine (AA) to some laboratory animals (e.g. rats) induces vascular lesions pathologically resembling human atherosclerotic disease. This organ-specific damage has been linked to the presence of a membrane-bound semicarbazide-sensitive amine oxidase (SSAO) in vascular smooth muscle cells, which metabolizes AA to the toxic aldehyde acrolein (ACR) [1]. Sources of direct human exposure to ACR include cigarette smoke, vehicle exhaust fumes, and its formation as a hepatic metabolite of the antineoplastic drug cyclophosphamide. Here, we have studied cytotoxic effects of AA and ACR upon human umbilical vein endothelial cells (HUVEC) in culture.

Confluent HUVEC (second-passage) in six-well plates were incubated with AA or ACR for 18 h in 1 ml culture medium. In some experiments, 100 µl of human umbilical artery (HUA) homogenate (tissue homogenised 2.5% w/v in 10 mM potassium phosphate buffer pH 7.8) was included as a source of SSAO to metabolize AA, since HUVEC lack this enzyme. Cell survival was then assessed by colorimetric assay determining the ability of living cells to metabolize a tetrazolium compound to a blue dye [2]. Total cell viability was unchanged by exposure to 5 or 10 µM ACR, but was significantly reduced to 17 ± 13% and 11 ± 8% of untreated controls by 50 and 100 µM ACR, respectively (means ± s.e. mean of five different cultures; $P < 0.05$). AA (10 µM) did not affect viability, whereas cell survival of 105 ± 4% with 50 µM AA, and 55 ± 6% with 100 µM AA, was decreased respectively to 26 ± 15% and 3 ± 1% when homogenate was included in the incubations ($n = 4$ cultures). In further experiments ($n = 4$), the low cell viability (19 ± 11%) following 50 µM AA + homogenate treatment was unchanged (19 ± 11%) when also including the monoamine oxidase (MAO) inhibitor, pargyline (100 µM), whereas the MAO/SSAO inhibitor propargylamine (100 µM) protected against this toxicity (95% ± 1% survival), presumably by preventing AA metabolism by the homogenate SSAO (and possibly also by a soluble SSAO activity present in the bovine serum used in the culture medium).

These results indicate that loss of integrity or function of the endothelial lining of blood vessels could arise from environmental exposure to ACR. Endothelial toxicity of AA here was attributable, at least in part, to its conversion to ACR by SSAO. Vascular SSAO can also metabolize the endogenous aliphatic amines methylamine and aminoacetone, respectively, to the potentially toxic aldehydes formaldehyde and methylglyoxal [3], although a role, if any, of these agents in mechanisms of vascular dysfunction remains to be established.

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1 Boor PJ, *et al. Circ Res* 1990; **66**: 249.
2 Mosmann T. *J Immunol Methods* 1983; **65**: 55.
3 Lyles GA. *J Neural Transm* 1994; [Suppl] **41**: 387.

Metabolism and covalent binding of furazolidone *in vitro* in pig liver and muscle fractions

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Furazolidone (FZ), a veterinary antimicrobial agent, is a known mutagen and a suspected carcinogen. Residues of such a compound in edible tissues could pose a health risk to the consumer. Covalently bound residues of FZ have been detected in pig tissue *in vivo* and studies using pig hepatocytes [1] have shown that metabolism and covalent binding of FZ can also occur *in vitro*. Metabolic activation of FZ has been shown to be necessary for the formation of FZ adducts in liver, however the issues concerning extrahepatic tissue involvement have not been investigated. Therefore we have examined factors affecting the formation of adducts of FZ in hepatic and extrahepatic tissue *in vitro* using pig liver and muscle fractions.

Two forms of [^{14}C]-radiolabelled FZ were obtained. FZ 'A' is labelled in the nitrofuran ring and FZ 'B' in the aminooxazolidone ring. This enabled the fate of both rings to be followed. The *in vitro* metabolism of FZ was studied using liver and muscle fractions prepared from tissue of female Duroc pigs ($n = 4$) and mean data ± s.d. reported. Incubation conditions were optimised for metabolism and covalent binding (50 µM FZ, 1 mg ml^{-1} protein, 15 min). These conditions were employed for all studies discussed unless stated otherwise. Metabolites of FZ were detected using

h.p.l.c. and radioflow detection set for [^{14}C]. The extent of covalent binding was quantified by successive washing and dissolving the protein for liquid scintillation counting. Any radioactivity associated with the dissolved protein was termed covalently bound. FZ metabolism was investigated in hepatic microsomes over a concentration range of 10–100 μM using both radiolabelled forms. Similar profiles were obtained for 'A' and 'B' with no statistical difference (paired *t*-test) at any of the concentrations studied. As the concentration of FZ increased the proportion covalently bound increased from 20% at 10 μM to 42% at 100 μM. Addition of 2 mM FAD to the incubation system produced a significant increase ($P < 0.005$) in both metabolism and covalent binding (8.9 ± 2.7 nmol to 82 ± 7 nmol and 3.1 ± 1.8 nmol to 8.9 ± 2.7 nmol respectively). No metabolism or covalent binding could be detected in the absence of NADP indicating that this cofactor is essential.

Metabolism and covalent binding of FZ by muscle microsomes or S9 could be detected only when FAD and NADP were both present. The amount of metabolites produced was 78 ± 4 nmol and 27 ± 4 nmol for microsomes and S9 respectively. Covalent binding for microsomes was 8.6 ± 1.9 nmol compared with 2.6 ± 1.1 nmol for S9. Although metabolism and covalent binding using S9 is significantly lower ($P < 0.005$) than with microsomes there is no significant difference between the ratio of metabolism to covalent binding for either of the two muscle fractions or the two tissue preparations.

This study indicates that provided FAD and NADP are present in tissue (e.g. muscle) then FZ is metabolised and covalently bound residues are formed. Also since no difference between the radiolabelled forms was detected this implies that residues resemble the parent compound, i.e. both rings are present.

1 Hoogenboom LA, *et al. Fd Chem Toxic* 1991; **29**: 321.

A comparison of the kinetics of caffeine and phenytoin metabolism in precision cut liver slices and freshly isolated hepatocytes

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We have investigated the metabolite kinetics of caffeine (CAFF) and phenytoin (DPH), both extensively used probe substrates for cytochrome P450 activity, in precision cut liver slices prepared from male Sprague-Dawley rats (250 g) using a Krumdieck Tissue Slicer. Following a 10 min preincubation, under conditions linear with respect to time and slice number, [^{14}C]-CAFF was incubated over a substrate range of 5–1000 μM and DPH over 0.5–100 μM. Following slice homogenisation, [^{14}C]-CAFF incubations were assayed using h.p.l.c. to separate metabolites from parent compound and total metabolism determined. DPH incubations were hydrolysed to release conjugated hydroxy DPH which was assayed by h.p.l.c. with u.v. detection.

Both CAFF and DPH metabolism displayed biphasic kinetics, the data being best described by a modified two site Michaelis-Menten equation. This comprised a high affinity, low capacity site (V_{max} 14.8 ± 4.2 and 76.4 ± 39.9 pmol min^{-1} slice^{-1}, K_m 12.1 ± 3.0 and 6.5 ± 3.5 μM and clearance (V_{max}/K_m) CL_1 1.2 ± 0.3 and 12.9 ± 4.4 μl min^{-1} slice^{-1} for CAFF and DPH respectively) and a low affinity, high capacity site (clearance CL_2 0.1 and 0.4 ± 0.3 μl min^{-1} slice^{-1} for CAFF and DPH respectively) which was not saturated over the concentration range studied. Total intrinsic clearance (CL_{int}) for CAFF and DPH metabolism was 1.3 ± 0.3 and 13.3 ± 4.4 μl min^{-1} slice^{-1} respectively (mean ± s.d., $n = 6$).

Various biochemical characteristics of our slices (258 ± 32 μm) were determined to allow comparison of kinetic parameters between this *in vitro* system and freshly isolated hepatocytes. The protein, DNA and cytochrome P450 content of slices were determined as 3.4 ± 0.4 mg, 60 ± 10 μg and 0.5 ± 0.1 nmol respectively (mean ± s.d., $n = 24$). Comparison with values determined in hepatocytes in our laboratory [1], allows the cell number of a slice to be estimated at 2.38×10^6.

The kinetics of CAFF and DPH in slices were expressed per 10^6 cells and compared with values determined in our laboratory in hepatocytes [1, 2]. For DPH the same model was necessary to describe the data in both *in vitro* systems, and the K_ms were in close agreement as was the case for tolbutamide and ethoxycoumarin previously reported by us [3]. In the case of CAFF, whereas a two site model was required for the slice data, a single site model was adequate with hepatocytes. A comparison of the CL_{int}s (per 10^6 cells) showed that slices metabolised at lower rates than hepatocytes (ratio of slice:cell CL_{int} = 0.13–0.26).

These results show that slices metabolise at least four drugs at substantially lower rates than isolated hepatocytes. Previously we have reported rates of metabolism to be proportional to slice thickness, and hence cell number. Therefore this phenomenon would appear to be related to restricted drug accessibility within the slice.

1 Carlile DJ. PhD. Thesis, University of Manchester, 1994.
2 Hayes KA, *et al. Drug Metab Dispos* 1995; in press.
3 Worboys PD, *et al. Drug Metab Dispos* 1995; in press.

In vitro interaction of (E)-5-(2-bromovinyl)uracil with 5-fluorouracil metabolism in human liver

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Dihydropyrimidine dehydrogenase (DPD) catalyses the initial step in the catabolism of the antineoplastic drug, 5-fluorouracil (FUra). Co-administration of sorivudine, an antiviral agent used to treat herpes zoster, with FUra has led to several fatalities which is thought to be due to the inhibition of FUra breakdown [1]. (E)-5-(2-bromovinyl)uracil (BVUra) is the major metabolite of sorivudine, (+)-1-B-D-arabinofuranosyl-5-(E)-bromovinyluracil [2]. BVUra reduces FUra degradation in rats and leukaemic mice *in vivo* and *in vitro* by inhibition of DPD activity [3]. In this study the effects of BVUra on the *in vitro* catabolism of FUra by human liver and rat DPD has been investigated.

Supernatant fractions (100,000 *g*) were prepared from male Sprague-Dawley rat livers or frozen human liver samples. Human DPD activity was characterised using FUra, 5-bromouracil and 5-iodouracil as substrates and 5-diazouracil as an inhibitor. Substrate reduction was monitored spectrophotometrically at 340 nm by measuring the decrease in absorbance of 0.12 mM NADPH. Incubations were performed at 37° C and also contained 1 mM dithiothreitol in a total volume of 3 ml 35 mM potassium phosphate buffer pH 7.4. It was necessary to pre-incubate BVUra or 5-diazouracil with 0.1 ml cytosol and NADPH for 10 min prior to the addition of FUra to achieve any inhibition of FUra breakdown. The effects of BVUra and 5-diazouracil on FUra metabolism in human liver fractions are shown in Table 1. Turnover of FUra by rat liver DPD (0.044 ± 0.038 μmol NADPH oxidised h^{-1} mg^{-1} protein) was higher than by human enzyme but BUVra still has a significant inhibitory effect on FUra reduction (0.006 ± 0.007 μmol NADPH oxidised h^{-1} mg^{-1}; 14% of control activity).

The results show that BVUra is an effective *in vitro* inhibitor of FUra catabolism by human DPD. It is thus likely that the potentiation of FUra toxicity by sorivudine co-administration is a result of DPD inhibition by its metabolite, BVUra.

Table 1 Activity of human liver DPD towards substituted pyrimidines and purines

Incubation	*μmol NADPH oxidised h^{-1} mg^{-1} protein*	*% Activity of FUra control*
5-Iodouracil (50 μM)	0.014 ± 0.003	—
5-Bromouracil (20 μM)	0.014 ± 0.004	—
FUra (20 μM)	0.026 ± 0.005	100
FUra + BVUra (20 μM)	0.009 ± 0.003**	35
FUra + 5-diazouracil (6 μM)	0.004 ± 0.004**	23

Results are expressed as mean activity ± s.e. mean (n = 3). **Reduction of FUra was significantly inhibited in the presence of BVUra and 5-diazouracil. $P < 0.05$ using an unpaired Student's t-test.

1 Swinbanks D. *Nature* 1994; **369**: 697.
2 Machida H, *et al. Antimicrob Agents Chemother* 1992; **36**: 214.
3 Desgranges C, *et al. Cancer Res* 1986; **46**: 1094.

Metabolism of the CYP2D substrates metoprolol and bufuralol by rat brain and liver microsomes

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Characterisation of the polymorphic cytochrome P450 sub-family, CYP2D in the brain is at an early stage. Using metoprolol and bufuralol as probe substrates, CYP2D activities were compared in brain and liver microsomes from male and female Wistar rats and from the female DA rat, a model of the human poor metaboliser phenotype.

Whole brain or liver microsomes were incubated with metoprolol or bufuralol at 37° C with a NADPH generating system. The appearance of α-hydroxymetoprolol (αHM) and *O*-demethylmetoprolol (ODM) and 1-hydroxybufuralol (1-OHB) was monitored by h.p.l.c.

ODM but not αHM was detected when metoprolol was incubated with brain microsomes from male Wistar rats. Since the amount of ODM formed, even at substrate concentrations up to 1000 μM, was close to the limit of detection of the assay (1.5 pmol min^{-1} mg^{-1}), no further kinetic analysis was performed with metoprolol. The kinetics of the 1-hydroxylation of bufuralol (50–1000 μM) by brain microsomes were monophasic and the apparent K_m and V_{max} values were similar in all rat strains (Table 1). 1-OHB was not detectable at bufuralol concentrations below 50 μM. The appearance of 1-OHB was only weakly inhibited by quinine (apparent K_i = 200 μM). Rat liver microsomes 1-hydroxylated bufuralol approximately 1000 times more rapidly than those from brain. The kinetics of this reaction (1–1000 μM) were biphasic in all three strains of hepatic microsomes. Values of

apparent K_{m1} and K_{m2} were similar in all strains (Table 1). However, V_{max} values of the high and low affinity sites in female DA were 10-fold and 3-fold lower, respectively, than those in Wistar microsomes. High affinity activity in all three strains was abolished by quinine (10 μM).

These findings confirm that at least two CYP enzymes mediate the 1-hydroxylation of bufuralol in rat liver microsomes, and indicate that the high affinity/low capacity site is CYP2D1. An isoform other than CYP2D1 catalyses bufuralol 1-hydroxylation in rat brain microsomes.

Table 1 Kinetic parameters for bufuralol 1-hydroxylation (mean ± s.d., n = 9 determinations from pooled microsomes)

	Rat liver microsomes				*Rat brain microsomes*	
Kinetic parameter	K_{m1} (μM)	V_{max1} (pmol min^{-1} mg^{-1})	K_{m2} (μM)	V_{max2} (pmol min^{-1} mg^{-1})	K_{m1} (μM)	V_{max1} (pmol min^{-1} mg^{-1})
Male Wistar	0.92 ± 0.20	1040 ± 11	53 ± 29	1140 ± 15	292 ± 25	1.14 ± 0.04
Female Wistar	0.83 ± 0.14	1345 ± 4	84 ± 20	1548 ± 7	202 ± 12	1.09 ± 0.04
Female DA	0.91 ± 0.26	117 ± 2	36 ± 17	405 ± 7	241 ± 16	1.17 ± 0.05

Ethacrynic acid modulates the *in vitro* cytotoxicity of mitoxantrone in MCF 7 human breast cancer cells

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The anticancer quinone drug mitoxantrone (MTX) is thought to be activated by cytochrome P450 dependent metabolism, and detoxified to a dihydroquinone by DT-diaphorase activity. Modulation of MTX toxicity by agents like ethacrynic acid (EA) which are known to increase DT-diaphorase activity *in vitro* may prove to be clinically useful. We have investigated the toxicity of MTX in MCF 7 human breast cancer cells in terms of total cell protein, reduced glutathione (GSH) content, leakage of lactate dehydrogenase (LDH) activity and viability measured by the MTT assay in the presence and absence of 10 μM EA.

MCF 7 cells were seeded at 5×10^4 cells/well on 24 well plates and when confluent (4 days post-passage) 10 μM EA was added. Fresh medium and EA were added daily for 3 days. On day 7 cells were exposed to 200 μM MTX in serum-free medium for 4 h before measurement of toxicity by the parameters indicated above. DT-diaphorase activity was also measured on day 7.

DT-diaphorase activity in control cells on day 7 was 0.43 ± 0.02 μmol min^{-1} mg^{-1} protein compared with 0.65 ± 0.01 μmol min^{-1} mg^{-1} protein in EA treated cells (n = 4; $P < 0.05$, by non-paired Student's t-test. Table 1 shows that pretreating cells with EA decreased the toxicity observed with MTX. It should be noted that EA treatment increased GSH content markedly. Therefore the amelioration of MTX toxicity by this agent could be due to a combination of increased DT-diaphorase activity and GSH content.

Table 1 The effect of EA on the toxicity of MTX

Pretreatment	*Total protein*	*LDH*	*GSH*	*Viability*
Control	1.89 ± 0.18	0.07 ± 0.01	1.29 ± 0.20	100 ± 4.7
10 μM EA	1.84 ± 0.14	0.04 ± 0.01	4.52 ± 0.29*	83.6 ± 2.1
200 μM MTX	1.14 ± 0.09*	0.26 ± 0.04*	0.33 ± 0.11*	25. 0 ± 2.8*
200 μM MTX plus 10 μM EA	1.68 ± 0.14**	0.18 ± 0.01**	1.43 ± 0.11**	71.4 ± 2.1**

Results are the mean ± s.e. mean of at least six experiments. Total protein is mg ml^{-1}; LDH is μmol min^{-1} mg^{-1} protein; GSH is μmol mg^{-1} protein and viability is % viable cells.
*$P < 0.05$, compared with control, and **$P < 0.05$, compared with 200 μM MTX. Statistical comparisons are by ANOVA, followed by Dunnett's test.

Zidovudine phosphorylation in peripheral blood mononuclear cells *in vitro*: effect of depleting T4 cells

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The anti-HIV drug zidovudine (ZDV) requires intracellular phosphorylation to its active triphosphate form in target lymphocytes. We have recently shown that concentrations of intracellular ZDV phosphates in isolated PBMCs are higher in more immunosuppressed patients with low CD4 (T4) counts [1] (Figure 1a). As PBMCs comprise different cell types (including B cells, T cells and monocytes) it is possible that the increase in ZDV phosphorylation may be explained by an altered cell population brought about by an increased viral load in these patients.

PBMCs were isolated by density cushion centrifugation from blood samples obtained from six HIV-negative individuals. T4 cells were separated by incubating PBMCs with magnetic polystyrene microspheres coated with T4 cell specific antibodies (Dynabeads) for 30 min at 4° C. Differing percentages of T4 enriched, T4 depleted and PBMC populations of cells were stimulated with 10 μg ml^{-1} phytohaemagglutinin (PHA) for 72 h followed by incubation with [^{3}H]-ZDV (0.65 μCi; 0.02 μM) for 5 h. Intracellular phosphates were extracted overnight with 60% methanol prior to analysis by anion exchange h.p.l.c.

T4 enriched cells phosphorylated ZDV to a significantly greater extent than a T4 depleted population of cells (Figure 1b). These results suggest that the increase in ZDV phosphates (particularly ZDV-monophosphate) seen in more immunosuppressed HIV+ patients (Figure 1a) cannot be explained by an altered ratio of cell type.

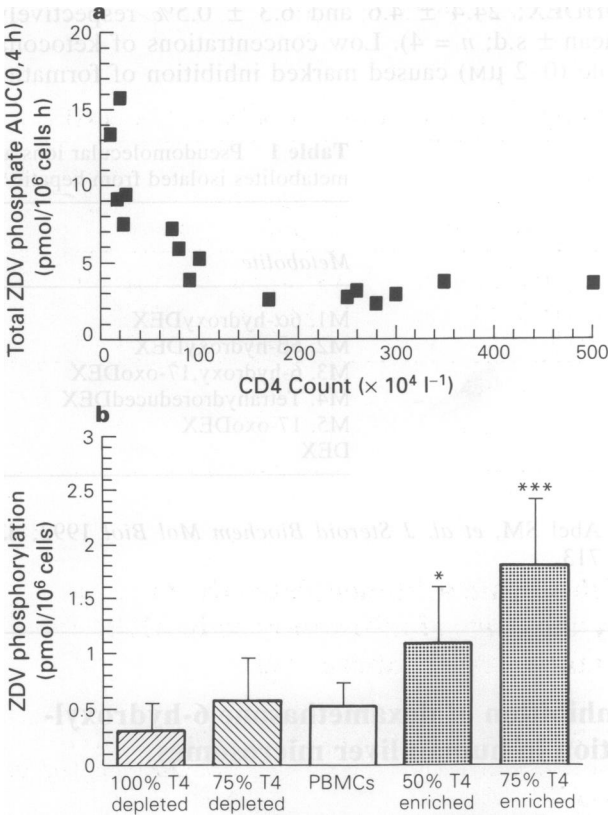

Figure 1 Intracellular phosphorylation of ZDV in a) 17 HIV+ patients and b) T4 enriched/depleted PBMCs. Results are mean ± s.d. (n = 6). Data analysed by ANOVA. *P < 0.05, ***P < 0.001 significantly different from both PBMCs and T4-depleted cells.

1 Barry M, *et al. AIDS* 1994; **8**: F1.

Dexamethasone metabolism by human liver *in vitro*: metabolite identification

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We have previously shown that the *in vivo* hepatic metabolism of the endogenous corticosteroid cortisol is complex [1], with at least eight metabolites identified by mass spectrometry (6α-hydroxycortisol, 6β-hydroxycortisol, 20β-dihydrocortisol, 20β-dihydrocortisone, cortisone, 3α,5β-tetrahydrocortisol, 3α,5β-tetrahydrocortisone, 6β-hydroxycortisone). Incorporation of a fluorine into the steroid (9α-fluorocortisol) greatly simplifies the metabolic profile in microsomes via extensive inhibition of 11β-dehydrogenation and 5β-reduction. Fluorocortisol was essentially metabolised by 20β-reduction and 6β-hydroxylation, alone [2]. In order to gain further insight into structure-biotransformation relationships we have examined the metabolism of the synthetic glucocorticoid dexamethasone (DEX), which in addition to possessing a 9α-fluorine has a 1,2-double bond and a 16α-methyl group.

Incubations at 37° C for 1 h contained [^{3}H]-DEX (0.1 μCi, 10 μM), $MgCl_2$ (10 μM), human liver microsomes (3 mg protein), NADPH (5 mM) and 0.067 M phosphate buffer (pH 7.4) to give a final volume of 0.5 ml. Extraction with ethyl acetate and diethyl ether was followed by radiometric and u.v. h.p.l.c. analysis. Metabolites were identified by co-chromatography

with authentic standards (when available) and h.p.l.c. mass spectrometry. Metabolites identified are shown in Table 1 which summarizes the mass spectral data.

DEX is metabolised in human liver microsomes by 6-hydroxylation (formation of both 6β- and 6α-OHDEX; 24.4 ± 4.6 and 6.3 ± 0.5% respectively; mean ± s.d; n = 4). Low concentrations of ketoconazole (0–2 μM) caused marked inhibition of formation of both isomers (IC_{50} < 0.9 μM); suggesting that CYP3A4 is the major isozyme involved. Two other metabolites (M3, 18.5 ± 4.4% and M5, 7.0%) were apparently formed by side chain cleavage followed by 17-oxidoreductase activity; M3 might be formed by hydroxylation of M5. A minor metabolite, M4, is a tetrahydro derivative of DEX. Unchanged DEX accounted for 43.6 ± 5.5%.

Table 1 Pseudomolecular ions and major ion fragments of dexamethasone metabolites isolated from hepatic *in vitro* studies

Metabolite	*Pseudomolecular (M+1) and fragment ions (relative intensity)*
M1. 6α-hydroxyDEX	409(M+1:100)
M2. 6β-hydroxyDEX	409(M+1:100), 345(56), 227(30)
M3. 6-hydroxy,17-oxoDEX	349(M+1:100), 142(21), 112(20)
M4. TetrahydroreducedDEX	397 (trace peak only seen)
M5. 17-oxoDEX	333(100), 311(34), 297(61), 279 (31)
DEX	393(100), 373(25)

1 Abel SM, *et al. J Steroid Biochem Mol Biol* 1992; **43**: 713.

2 Abel SM, *et al. J Steroid Biochem Mol Biol* 1993; **46**: 833.

Inhibition of dexamethasone 6-hydroxylation in human liver microsomes

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There is evidence from both *in vitro* and *in vivo* studies that the synthetic glucocorticoid dexamethasone (DEX) is hydroxylated to both 6α- and 6β-hydroxyDEX [1, 2]. We have used a number of selective inhibitors and substrates of different cytochrome P450 (CYP450) isozymes to assess which isozyme is primarily responsible for the 6-hydroxylation of the steroid in human liver microsomes.

[^{3}H]-DEX (1–100 μM, 0.1 μCi) was incubated at 37° C with microsomal protein (3 mg), $MgCl_2$ (100 mM), NADPH (5 mM) and a range of potential inhibitors (0–100 μM) in a final incubation volume of 0.5 ml for 60 min. DEX and its metabolites were extracted and analysed by radiometric h.p.l.c. (column: Nucleosil $5C_8$ (5 μm, 25 cm × 4.6 mm i.d.); mobile phase: 0.5% ammonium orthophosphate:acetonitrile, 75:25 v/v, 0.7 ml min^{-1}). The mean K_m and V_{max} values for overall 60HDEX formation determined from four livers were 37 ± 6.2 μM and 14.1 ± 2.2 pmol min^{-1} mg^{-1} microsomal protein respectively. Ketoconazole (a selective inhibitor of CYP3A4 at low concentration) gave the most potent inhibition (K_i < 1.0 μM) consistent with previous findings of inhibition of cortisol 6β-hydroxylase *in vitro* [3]. Both 6α- and 6β-OHDEX declined in parallel in the presence of ketoconazole. Gestodene (a proposed mechanism-based inactivator and substrate of CYP3A4) demonstrated potent inhibition as did the alleged CYP1A inhibitor ellipticine. Another specific CYP1A inhibitor (furafylline) was not inhibitory indicating that ellipticine is not selective for CYP1A. Cortisol and fluconazole also showed some inhibitory potential. Specific substrates and inhibitors of other CYP isozymes (e.g. theophylline, CYP1A2; tolbutamide and sulphaphenazole, CYP2C9) were not inhibitory thus indicating the substrate specificity of DEX and CYP3A4 (Table 1). Dexamethasone may prove to be a useful probe for CYP3A4.

Table 1 Effect of CYP450 substrates and inhibitors on the 6-hydroxylation of DEX in human liver microsomes

Compound	IC_{50} (μM)	K_i (μM)
Ketoconazole	0.48 ± 0.08	0.73 ± 0.44
Fluconazole	31.7 ± 21.1	ND
Gestodene	10.3 ± 4.5	14.7 ± 6.4
Cortisol	81.3 ± 8.1	ND
Tolbutamide	>100†	ND
Sulphaphenazole	>100†	ND
Theophylline	>100†	ND
Ellipticine	4.43 ± 1.5	2.53 ± 0.87
Furafylline	>100†	ND

Values represent the mean ± s.d. of three individual determinations except † where n = 2.
ND = not determined.

1 Minagawa K, *et al. Steroids* 1986; **47**: 175.

2 Tomlinson ES, *et al. Br J Clin Pharmacol* 1995; in press.

3 Abel SM, Back DJ. *Steroid Biochem Mol Biol* 1993; **46**: 827.

Use of the Comet assay to detect activation of 2-amino-3-methylimidazo (4,5-*f*) quinoline (IQ) in V79 cells expressing CYP1A2 and acetyltransferase

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IQ is a heterocyclic aromatic amine produced in the cooking of food and is a rodent carcinogen. Previous studies have implicated the role of cytochrome P450 1A2 in the metabolic activation of this pro-mutagen to *N*-hydroxylated IQ which is then esterified (e.g. by acetylation) to produce a DNA-reactive product [1].

We have assessed the ability of IQ to induce DNA strand breakage in cultured Chinese hamster V79 cells (V79NHr1A2) genetically engineered to express rat CYP1A2 and acetyltransferase. Strand breakage was measured by single cell gel electrophoresis (Comet assay, based on Singh *et al.* [2]) in cells during log-growth phase. The migration of DNA from the nucleus (comet) was assessed by microscopy and image analysis. Twenty-five cells were analysed in triplicate. The ability of 9,000 *g* supernatants from these cells to convert IQ into mutagenic species was confirmed by demonstrating the formation of mutagens detected by *Salmonella typhimurium* strain TA98.

The tail moment of comets (related to extent of DNA damage) increased from 1.8 ± 0.2% (mean ± s.d., n = 3 control) to 13.5 ± 2.1% following incubation with IQ (25 μM, 1 h). An increase in tail moment was observed at 2.5 μM and above whereas no effect was observed in parental V79 cells devoid of cytochrome P450 and acetyltransferase activity. The percentage of V79NHr1A2 cells demonstrating appreciable damage (>20% DNA in the comet tail) was 67% at 25 μM IQ compared with 10% in controls. The breakage of DNA strands may either be direct or mediated by excision enzymes responsible for repair of DNA adducts derived from IQ.

The results demonstrate the potential value of genetically engineered cells to study the role of specific enzymes involved in the activation of pro-genotoxic chemicals [3].

Funds were provided by the EU. Contract EV5V-CT91-0006.

1 Sugimura T, Wakabayashi K. *Adv Exp Med Biol* 1991; **283**: 569.
2 Singh NP, *et al. Exp Cell Res* 1988; **175**: 184.
3 Doehmer J. *Toxicology* 1993; **82**: 105.

Sulphoraphane exhibits cytochrome P4502E1-mediated activation of dimethylnitrosamine

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Sulphoraphane (1-isothiocyanate-4-methylsulphinylbutane) is a constituent of broccoli which protects against rodent tumourogenicity of 9,10-dimethyl-1,2-benzanthracene, possibly via its ability to induce glutathione-S-transferase [1, 2]. Based on the knowledge that related isothiocyanates also inhibit tumour formation in rodents treated with nitrosamines, we have now tested the hypothesis that sulphoraphane also inhibits cytochrome P4502E1 and thus could reduce genotoxicity of agents activated by this enzyme.

Sulphoraphane inhibited the hydroxylation of *p*-nitrophenol (P4502E1 substrate) by liver microsomes from acetone-treated Sprague Dawley rats (K_i, 37.0 ± 4.5 μM). Enzyme activity was assessed spectrophotometrically by measurement of the hydroxylation of *p*-nitrophenol to *p*-nitrocatechol [3]. Sulphoraphane also inhibited the genetic toxicity of *N,N*-dimethylnitrosamine (DMNA, P4502E1 substrate). DMNA (4.4 mg/plate) was incubated (45 min) with *Salmonella typhimurium* strain TA100 in the presence or absence of sulphoraphane and hepatic postmitochondrial supernatant from acetone-pretreated Balb/c mice. Mutagenicity was inhibited by sulphoraphane (>0.8 μM) (73.4% inhibition at 200 μM). DNA repair as measured by unscheduled DNA synthesis produced by DMNA (33.5 μM) in Balb/c mouse hepatocytes was also inhibited by sulphoraphane (>0.06 μM) (92% inhibition at 20 μM). In the assays employed, sulphoraphane was itself non-genotoxic and non-cytotoxic. It was also not able to inhibit the bacterial mutagenicity of a direct acting mutagen (sodium azide, 5 μg/plate).

The results suggest that inhibition of cytochrome P4502E1 contributes to antigenotoxicity and (coupled with its ability to induce protective phase 2 enzymes) further implicate sulphoraphane as a potential chemopreventive agent.

1 Zhang Y, *et al. Proc Natl Acad Sci* 1992; **89**: 2399.
2 Zhang Y, *et al. Proc Natl Acad Sci* 1994; **91**: 3147.
3 Reinke LA, Moyer MJ. *Drug Metab Dispos* 1985; **13**: 548.

Structural requirements for the inhibition of CYP2D6 by imipramine

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There are several models for the CYP2D6 active site with the characteristics of their substrates and inhibitors defined [1, 2]. Characteristics of inhibitors and substrates include: a positively charged nitrogen atom, groups containing negative potential positioned 5–7 Å from this nitrogen; a flat lipophilic region and functional groups which have the capacity for electrostatic interactions or the ability to form hydrogen bonds.

The tricyclic antidepressant imipramine (Figure 1) possesses several of these characteristics and is an inhibitor of bufuralol 1'-hydroxylation (10 μM), an *in vitro* marker for CYP2D6 enzyme activity. In one human liver genotyped and phenotyped for CYP2D6 as an extensive metaboliser (EM) and one as a poor metaboliser (PM) for CYP2D6, imipramine exhibited differential inhibition of bufuralol 1'-hydroxylation producing IC_{50}s of 26.1 ± 4.2 μM and 114.9 ± 19.6 μM respectively suggesting selectivity towards CYP2D6. Data shown is mean ± s.e. mean for quadruplicate incubations.

Further studies, with the EM liver, examined the effect chemical modification of the imipramine molecule has on the inhibitory potency against CYP2D6.

Both an increase in the N-N chain length from 3 to 4 (IC_{50} = 98.4 ± 13.0 μM) and a decrease to a 2-carbon bridge (IC_{50} = 112 ± 16.1 μM) decreased inhibitory potency.

Introduction of a carbonyl group, which restricts rotation of the side chain, into analogues with a 2 (IC_{50} > 1 mM), 3 (IC_{50} = 121.8 ± 41.2 μM) and 4 (IC_{50} > 500 μM) carbon bridge, in each case brought about a reduction in inhibitory potency.

Finally, modification of the terminal nitrogen by either addition of a methyl group (IC_{50} > 1 mM), or removal of methyl (IC_{50} = 64.4 ± 15.9 μM), or *N*-oxidation (IC_{50} > 1 mM), all reduced potency. There was found to be no clear relationship between physicochemical properties and inhibitory potency.

Thus, although most compounds retained some inhibitory activity, any chemical modification resulted in some loss of potency, indicating that stereochemical factors are an important determinant of the ability of this class of compound to interact with CYP2D6. The loss of inhibitory potency of the *N*-oxide, as a result of the reduction of positive charge on the nitrogen and possible steric hinderance, illustrates the importance of the ion pairing with a corresponding negative charge on the CYP2D6 active site.

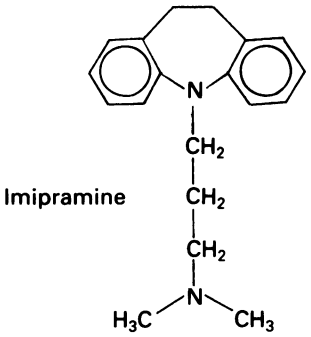

Figure 1

1 Koymans L, *et al. Chem Res Toxicol* 1992; **5**: 211.

2 Strobl GR, *et al. J Med Chem* 1993; **36**: 1136.

Toxicity of the bioreductive drug SR 4233 (tirapazamine) is determined by P450 reductase activity in human breast but not lung cancer lines

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SR 4233 (3-amino-1,2,4-benzotriazine-1,4-di-*N*-oxide, tirapazamine) is a bioreductively activated hypoxic cell cytotoxin. The cytotoxicity arises as a consequence of DNA damage caused by the one electron reduced di-*N*-oxide. Studies using liver microsomes [1] and tumour cells [2] have shown that NADPH: P450 reductase is an important enzyme in the reductive activation of SR 4233.

In this work we have investigated the importance of NADPH: P450 reductase in determining the toxicity of SR 4233 in a panel of six human breast adenocarcinoma cell lines. Cells grown in culture were exposed to SR 4233 for 3 h in both air and under nitrogen. Drug sensitivity was determined using the MTT assay [3]. NADPH: P450 reductase activity was measured as the NADPH-dependent reduction of cytochrome c catalysed by lysates prepared from the cell lines. Reduction of SR 4233 to the stable 2-electron reduced product, SR 4317, was determined by incubating cell lysates with the parent drug (2 mM), NADPH (1 mM) at 37° C under nitrogen for 40 min. Formation of SR 4317 was determined by h.p.l.c.

In the panel of cell lines IC_{50} values (SR 4233 concentration reducing cell survival to 50% of untreated

controls) following acute 3 h hypoxic exposure ranged from 5.1 ± 2.0 to 23.8 ± 4.0 μM (mean ± s.d.; n = 4 incubations). A highly significant correlation was observed between the sensitivity of the cell lines to SR 4233 and P450 reductase activity which varied from 6.9 ± 1.8 to 39.8 ± 3.2 nmol cytochrome c reduced min^{-1} mg^{-1} protein (mean ± s.d.; n = 4 to 6) ($P < 0.001$). In contrast, no correlation was observed between P450 reductase activity and IC_{50} following acute aerobic exposure (IC_{50} values: 0.17 ± 0.48 to 1.30 ± 0.5 mM; mean ± s.d.).

Rates of formation of SR 4317 by cell lysates ranged from 3.7 ± 0.7 to 22.4 ± 3.4 nmol min^{-1} mg^{-1} protein (mean ± s.d., n = 3 lysates from each cell line). Significant correlations between SR 4317 formation velocity and both P450 reductase activity ($P < 0.01$) and drug toxicity following acute hypoxic exposure ($P < 0.04$) were observed, the drug having greatest toxicity in the cell line with the highest P450 reductase activity and highest rate of SR 4317 formation.

Similar studies were performed using a panel of seven human lung cancer cell lines, derived from a number of different histological types. Whilst values for P450 reductase activity, SR 4317 formation and toxicity of SR 4233 were similar to those observed in the breast cell lines, no correlations were observed between toxicity and P450 reductase activity or SR 4317 formation.

These data indicate that P450 reductase plays a major role in determining the hypoxic toxicity of SR 4233 in human breast cancer cell lines, presumably by activation of the drug to a toxic free radical. In contrast, in the lung lines P450 reductase appears to be of little or no relevance in determining drug toxicity or in the activation of SR 4233. The mechanistic basis for these differences between the breast and lung cells is currently under investigation.

1 Lloyd RV, *et al. Mol Pharmacol* 1991; **40**: 440.
2 Wang J, *et al. Br J Cancer* 1993; **67**: 321.
3 Carmichael J, *et al. Cancer Res* 1987; **47**: 936.

Halofantrine is a substrate of cytochrome P4503A (CYP3A)

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The antimalarial halofantrine possesses typical characteristics of CYP2D6 substrates and inhibitors [1]. It was therefore of interest to investigate whether there was an interaction between the polymorphic CYP2D6 enzyme and this drug. Halofantrine was found to be a potent inhibitor of the *in vitro* l'-hydroxylation of bufuralol in human liver microsomes, an oxidation catalysed by CYP2D6, but inhibition studies with selective low molecular weight inhibitors and kinetic studies with livers genotyped for CYP2D6 status, suggested that halofantrine is preferentially metabolised to *N*-desbutyl halofantrine by an enzyme other than CYP2D6 [2].

The potent inhibition by ketoconazole (IC_{50} = 1.57 ± 0.67 μM, at 100 μM halofantrine and total inhibition at 100 μM ketoconazole) indicated that CYP3A effected halofantrine metabolism.

The role of CYP3A in the *N*-debutylation of halofantrine has therefore been investigated further, by correlation studies with CYP3A protein levels and marker substrate activities.

Microsomal CYP3A protein levels were analysed by SDS-PAGE and immunoblotting with antisera obtained from rabbits immunised with a synthetic human CYP3A protein in seven human livers [3].

Levels of functional CYP3A in these seven livers were measured by the rate of felodipine oxidation to its pyridine, a probe reaction for this CYP3A isozyme [4].

There was found to be significant correlation between the rate of halofantrine *N*-debutylation in these livers, with both CYP3A protein levels (r = 0.88, P = 0.008) and the rate of felodipine oxidation (r = 0.89, P = 0.008) (Table 1).

The selective metabolism of halofantrine by CYP3A therefore implies that *in vivo* the inter-individual variation in both activity and level of expression of this enzyme, in both the liver and gut wall [5, 6], may contribute to the variable disposition of halofantrine.

Table 1 The rate of metabolite formation for both halofantrine (100 μM) and felodipine (100 μM) are given in pmol product min^{-1} mg^{-1} microsomal protein and represent the means of quadruplicate incubations

Liver	N-*desbutyl halofantrine* (± s.d.)	*Felodipine pyridine* (± s.d.)	*CYP3A levels (volume densities)*
L7	32.8 ± 2.5	707.3 ± 42.3	1882
L17	22.8 ± 1.6	637.7 ± 22.0	1123
L18	17.4 ± 0.9	436.6 ± 8.0	1028
L19	20.1 ± 0.8	481.4 ± 7.4	930.5
L20	27.2 ± 1.1	544.4 ± 8.7	1671
L22	23.9 ± 6.1	502.0 ± 16.6	1469
L36	34.0 ± 8.6	839.2 ± 14.3	3073

1 Smith DA, Jones BC. *Biochem Pharmacol* 1992; **44**: 2089.
2 Halliday RC, *et al. Br J Clin Pharmacol* 1995; **39**: 556P.
3 Hyland R, *et al. Br J Clin Pharmacol* 1995; **40**: 179P.
4 Guengerich FP. *J Med Chem* 1991; **34**: 1838.
5 Kolars JC, *et al. J Clin Invest* 1992; **90**: 1871.
6 Forester LM, *et al. Biochemistry* 1992; **281**: 359.

Immunochemical detection of covalently modified hepatic proteins produced following administration of methyleugenol to rats

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Methyleugenol (ME) is a naturally occurring hepatotoxin and carcinogen [1]. As well as covalently binding to DNA, ME covalently binds to a number of hepatic proteins which may play a role in the toxicity of ME. In the present study we have used immunochemical techniques to investigate the nature and subcellular location of covalently modified proteins formed in rat liver following i.p. dosing with ME.

Male Fischer 344 rats (200–250 g; n = 5 per group) were dosed with ME at either 0, 10, 30, 100 or 300 mg kg^{-1} day^{-1} for 5 days and then sacrificed. The livers were removed, homogenised and pooled. Nuclear (100 g for 3 min), mitochondrial (10 000 g; 20 min), microsomal (100 000 g; 60 min) and cytosolic (100 000 g supernatant) fractions were prepared by differential centrifugation. ME-protein adducts in these fractions were identified by enzyme linked immunosorbent assay (ELISA) and immunoblotting [2] using antisera specific for ME-adducts. The antisera were obtained from rabbits immunised with a conjugate produced by reacting 1'-acetoxymethyleugenol with RSA (primary immunisation 0.5 mg conjugate in Freund's complete adjuvant and then boost after 4 weeks with 0.5 mg in Freund's incomplete adjuvant). ELISA analysis showed that the sera recognised the ME-RSA conjugate. Inhibition studies demonstrated the specificity of the antisera, showing that they recognised ME, isomethyleugenol and the related compound dimethoxycinnamic acid but not other alkenylbenzene compounds.

ELISA studies revealed a dose-dependent formation of ME-covalently modified proteins in the ME treated rats ($P < 0.05$ as determined by ANOVA) (Table 1). High levels of covalent modification were detected in microsomal, mitochondrial and nuclear fractions. Immunoblot studies showed that more than 20 polypeptides were covalently modified by ME. The major targets were a 44 kDa (predominately unclear) protein and a 42 kDa (predominantly microsomal) protein and were detectable at all dose levels. Work is underway currently to identify these proteins and to assess their possible roles in the toxicity and carcinogenicity of ME.

This study was supported by MAFF contract 1A008.

Table 1 ELISA analysis of subcellular fractions prepared from livers of ME dosed animals (A_{492}; mean ± s.d.; n = 3 replicate determinations)

	Control	*10 mg kg^{-1}*	*30 mg kg^{-1}*	*100 mg kg^{-1}*	*300 mg kg^{-1}*
Whole homogenate	0.34 ± 0.04	0.38 ± 0.04	0.60 ± 0.03	1.01 ± 0.05	2.18 ± 0.13
Nuclear	0.51 ± 0.05	0.54 ± 0.04	0.72 ± 0.11	1.27 ± 0.05	2.90 ± 0.10
Mitochondrial	0.44 ± 0.07	0.54 ± 0.04	0.74 ± 0.05	1.39 ± 0.05	2.06 ± 0.05
Microsomal	0.59 ± 0.01	0.67 ± 0.06	0.99 ± 0.07	1.94 ± 0.10	3.39 ± 0.05
Cytosolic	0.23 ± 0.03	0.24 ± 0.02	0.35 ± 0.01	0.60 ± 0.03	1.34 ± 0.04

1 Miller EC, *et al. Cancer Res* 1983; **43**: 1124.

2 Kenna JG, *et al. Ann NY Acad Sci* 1993; **685**: 646.

Topical *in vitro* absorption of [^{14}C]-coumarin in human skin

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The naturally occurring plant alkaloid, coumarin (1,2-benzopyrone) is widely used as a fragrance chemical [1] and is also currently under investigation as a therapeutic agent. Clinically, it has been employed for the treatment of a range of chronic infections and inflammatory conditions such as brucellosis and lymphoedema, and it is also under investigation as a drug for the treatment of cancers such as metastatic renal cell carcinoma [2] and malignant melanoma [3]. Since coumarin may be applied to the skin in various preparations such as drugs and consumer products, it is important to establish its rate and extent of absorption (bioavailability) through human skin.

The percutaneous absorption of [4-^{14}C]-coumarin has been studied using fresh, metabolically viable, full-thickness human breast skin (obtained from surgical procedures) from two individuals aged 66 (subject 1) and 54 years (subject 2). The skin from each individual was cut into 14 circles and placed into diffusion cells of an *in vitro* Skin Absorption Model (SAM) previously validated as a representative model

of *in vivo* percutaneous absorption [4]. After exposure of the skin surface (0.32 cm^2) to 5 µl coumarin (3.7 µg cm^{-2} in ethanol), the surface was either left open to the atmosphere or covered (occluded) with a teflon cap. The receptor fluid (Hepes-buffered Hanks balanced salt solution supplemented with 0.5% gentamicin), which flowed under the skin at 1.5 ml h^{-1}, was collected for up to 72 h and was assayed for absorbed radioactivity by liquid scintillation spectrometry. At 72 h, the skin was swabbed and digested in methanolic sodium hydroxide/Triton X405 and assayed for residual radioactivity.

After topical application of coumarin to unoccluded human skin, the absorption into the receptor fluid over 72 h was rapid and extensive, reaching 45.6 ± 4.6% (mean ± s.d., n = 7 replicates) of the applied dose for subject 1, and 57.4 ± 9.5% (mean ± s.d., n = 7) of the applied dose for subject 2. When the skin was occluded, the absorption of coumarin at 72 h was 59.6 ± 5.7% (n = 7 replicates) of the applied dose for subject 1, compared with 75.6 ± 6.3% (n = 7) of the applied dose for subject 2. The residual radioactivity recovered from within the skin at 72 h was: 9.9 ± 3.7% (occluded) and 11.5 ± 6.8% (unoccluded) for subject 2.

Taken together, these data indicate that coumarin is well absorbed into human skin with a total uptake (skin plus receptor fluid) of up to 80% of the applied dose. Hence, in the absence of any cutaneous metabolism, high systemic bioavailability of coumarin would be expected after skin application in humans *in vivo*. We are currently investigating the cutaneous metabolism of coumarin, in order to establish whether systemic exposure is to the parent compound and/or to its metabolites.

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1 Cohen AJ. *Fd Chem Toxic* 1979; **17**: 277.
2 Marshall ME, Mohler JL. *J Irish Coll Phys Surg* 1993; **22**: suppl. 1, 6.
3 Thornes RD, *et al. Lancet* 1982; **ii**: 328.
4 Hotchkiss SA, *et al. Fd Chem Toxic* 1990; **28**: 443.

A high-sensitivity assay for the determination of RP 73401 in human plasma using h.p.l.c. with on-line fluorescence detection

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RP 73401 (3-cyclopentyloxy-*N*-(3,5-dichloro-4-pyridyl)-4-methoxybenzamide) is a selective inhibitor of phosphodiesterase (PDE) type IV, and *in vivo* studies have shown it to be a potent bronchodilator with anti-inflammatory properties. It is currently being evaluated as a potential antiasthmatic agent.

A high sensitivity h.p.l.c. method, with fluorescence detection, capable of determining human plasma RP 73401 concentrations in the pg ml^{-1} range, has been developed. This has been used to monitor RP 73401 plasma levels in individuals involved in early clinical studies with this product. RP 73401 was added to 1 ml plasma aliquots to give eight concentrations in the range 10 to 2000 pg m l^{-1}; the internal standard (RP 68090; 2000 pg) was added to each aliquot, and the samples were then extracted with 5 ml spectroscopic grade diethyl ether. After vortex mixing for 10 s, the samples were centrifuged at 1500 rev min^{-1} (RCF = 500) for 5 min. The organic phase was then removed and evaporated to dryness under a stream of nitrogen at 40° C. The residue was dissolved in 200 µl of the h.p.l.c. mobile phase and aliquots (180 µl) injected onto the h.p.l.c. The analytical column used was a 125 × 4 mm i.d. LiChroCART cartridge (Merck Ltd), packed with LiChrospher RP18 (5 µm particle size). The mobile phase used was acetonitrile:methanol: water, 41:14:45 by vol., at a flow rate of 1 ml min^{-1}, resulting in a back pressure of *circa* 700 psi. After passage through the column, and prior to entering the fluorescence detector, the column effluent was passed through a BeamBoost photochemical reactor, fitted with a 254 nm u.v. lamp, and a 20 m PTFE coil. The fluorescence of the resulting products was measured using excitation and emission wavelengths of 300 and 400 nm, respectively.

The method has been shown to be linear in the range 10 to 2000 pg ml^{-1}, with a limit of accurate quantification of 10 pg ml^{-1}, and a limit of detection of *circa* 2 pg ml^{-1}. The accuracy and precision of the assay was assessed on each of 3 days, and both inter- and intra-assay accuracy and precision were within acceptable limits (accuracy: mean calculated concentration = 90 to 115% of nominal, precision: %CV < 20 at 10 pg ml^{-1}, and < 10 at the other three levels studied (20, 200 and 2000 pg ml^{-1})).

The method has been applied to the assay of plasma samples obtained from a number of individuals given a single inhaled dose of 50 µg RP 73401, along with those from individuals in the same study who had received placebo. Using this assay, the drug could be quantified from 2.5 min to at least 12 h, and on occasion 24 h post-dose. No peaks which would interfere with the assay were detected, either in pre-dose plasma samples obtained from active-dosed subjects, or in any plasma sample assayed from placebo-dosed subjects.

Pharmacokinetics of intravenous fluticasone propionate in healthy subjects

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Fluticasone propionate (FP) is a potent topical glucocorticosteroid used in the treatment of asthma and allergic rhinitis. Pharmacokinetics of intravenous FP have been investigated, in a cross-over study, following single 250, 500 and 1000 μg doses (3 min infusions) in 12 healthy male volunteers, mean age 28 years (range 21–38 years) and mean weight 75 kg (range 55–100 kg). Local Ethics Committee approval was obtained prior to starting. Blood samples for plasma FP determination, were taken predose, 3, 4, 5, 6, 8, 10, 15, 20, 30, 45 min and 1, 2, 3, 4, 6, 8, 12, 16, 20 and 24 h post-dose. They were analysed using a radioimmunoassay [1].

Dose-normalised plasma concentration-time profiles following the three different doses were superimposable, indicating the pharmacokinetics of FP were proportional to dose over the 250 to 1000 μg dose range investigated. The pharmacokinetic profile of intravenous FP could be described by three exponentials (Table 1). However, the 250 μg dose was best described by two exponentials as the plasma concentrations associated with the third exponential term were generally below the assay's lower limit of quantification (0.05 ng ml^{-1}). This resulted in systemic exposure (AUC) being underestimated and plasma clearance being overestimated following the 250 μg dose. There was good agreement between the non-compartmental analysis and the exponential fitting. Accurate pharmacokinetic parameters were then determined by averaging the 500 and 1000 μg doses from the non-compartmental analysis which was considered to be more robust. FP was extensively distributed within the body (V_{ss} 318 l) and rapidly cleared (CL 1.1 l min^{-1}). Peak plasma concentrations were reduced by approximately 98% within 3–4 h. The terminal elimination half-life was 7.8 h. Because of the polyexponential kinetics only low plasma concentrations (<0.2 ng ml^{-1}) were associated with the long terminal elimination half-life.

1 Bain BM, *et al. J Pharm Biomed Analysis* 1993; **11**: 557.

Table 1 Pharmacokinetic parameters of intravenous fluticasone propionate (geometric mean 95% CI)

	Exponential fitting						*Non-compartmental analysis*			
Dose (μg)	$t_{1/2}(\lambda_1)$ *(h)*	$t_{1/2}(\lambda_2)$ *(h)*	$t_{1/2}(\lambda_z)$ *(h)*	*MRT (h)*	*CL (l min^{-1})*	V_{ss} *(l)*	C_{max} *(ng ml)*	t_{max}* *(h)*	*AUC (ng ml^{-1} h)*	$t_{1/2}$ *(h)*	*CL (l min^{-1})*	V_{ss} *(l)*
1000	0.03 (0.03–0.04)	0.92 (0.6–1.4)	7.92 (5.71–10.98)	4.27 (3.58–5.08)	1.05 (0.93–1.17)	268 (219–329)	63.7 (50.2–80.8)	0.06 (0.05–0.07)	14.5 (12.9–16.4)	7.15 (5.50–9.37)	1.15 (1.02–1.29)	287 (233–354)
500	0.03 (0.02–0.04)	1.03 (0.61–1.74)	8.47 (5.04–14.24)	5.09 (3.28–7.89)	0.95 (0.82–1.11)	290 (191–441)	32.8 (25.0–42.9)	0.07 (0.05–0.08)	8.0 (6.9–9.3)	8.45 (5.46–13.08)	1.04 (0.90–1.21)	349 (238–510)
250	0.04 (0.03–0.06)	1.84 (1.49–2.27)	—	2.12 (1.51–2.96)	1.20 (1.05–1.37)	152 (111–209)	12.8 (8.8–18.6)	0.07 (0.05–0.1)	3.3 (2.9–3.7)	3.43 (2.13–5.50)	1.26 (1.11–1.44)	213 (144–315)

*Median and ranges.